RESEARCH NOTE
Laboratory Bioassays on the Susceptibility of Trimen's False Tiger Moth, *Agoma trimenii* (Lepidoptera: Agaristidae), to Entomopathogenic Nematodes and Fungi

C. Morris[1], A.P. Malan[1], J.Y. De Waal[2], S. Johnson[1,*]

(1) Department of Conservation Ecology and Entomology, Faculty of AgriSciences, Stellenbosch University, Private Bag X1, Matieland 7602, Stellenbosch, South Africa
(2) Corteva Agriscience, Block A, 2nd Floor, Lakefield Office Park, 272 West Avenue, De Howes, Centurion, Gauteng 0157, South Africa

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Trimen's false tiger moth, *Agoma trimenii* (Lepidoptera: Agaristidae), recently developed as a pest of grapevine in the Northern Cape and Limpopo (Groblersdal area) provinces of South Africa. Little is known about the biology of *A. trimenii* and control options are lacking. The aim of this study was to test the susceptibility of *A. trimenii* larvae and pupae to two locally isolated entomopathogenic nematodes (EPNs), *Steinernema yirgalemense* and *Heterorhabditis noenieputensis*, and two commercially available entomopathogenic fungi (EPF), *Metarhizium anisopliae* and *Beauveria bassiana*, under laboratory conditions. Larvae and pupae were screened for pathogenicity of the two nematode species, using a concentration of 100 infective juveniles (IJs)/50 µl of water. After 48 h, 100% mortality of the larval stage was found. However, no pupae were infected with EPNs. Larvae and pupae were screened for pathogenicity of the two EPF isolates by means of a dipping test, at a concentration of 0.2 ml/500 ml water and 0.5 g/500 ml water, respectively. Five days post-treatment, 100% larval mortality was recorded in comparison with no deaths in the controls. Overt mycosis was only observed in the case of *M. anisopliae*. However, in the case of pupae, no mortality was observed for both the nematode and the fungal applications. In future studies, the prepupal soil stage of *A. trimenii* should be screened for susceptibility to EPNs and EPF. The results of this study indicate the excellent potential of EPNs and EPF as biological control agents against the larvae of *A. trimenii*, especially for application to small areas with high infestation, without disrupting an integrated pest management programme.

INTRODUCTION

Trimen's false tiger moth, *Agoma trimenii* Felder, is an indigenous lepidopteran that is classified within the Noctuidae family and the subfamily Agaristidae. *Agoma trimenii* targets the young shoots and leaves of wine, table and raisin grapes in the Northern Cape and Limpopo provinces of South Africa. As challenges associated with *A. trimenii* in the grape-producing regions of South Africa increase, so does the need to reduce populations to below damaging levels. Since the occurrence of *A. trimenii* is sporadic and confined, it is important to be able to control seasonal spikes in an environmentally friendly way, without disrupting natural enemies and the current control measures applied in vineyards (Morris, 2019; Morris *et al*., 2020). Biocontrol agents are a safe alternative, as they can be applied at any time close to or during harvest.

Potential biological control strategies for *A. trimenii* include the application of entomopathogenic nematodes (EPNs) and entomopathogenic fungi (EPF). Such biological control agents exhibit many beneficial traits, including offering minimal risk to human health, the absence of toxic residues in crops, minimal risk to beneficial and other non-target insects, and host specificity (Inglis *et al*., 2001; Goettel *et al*., 2005). Testing the above-mentioned biological control options on *A. trimenii* is important, as no such control options currently exist.

EPNs belonging to the genera *Heterorhabditis* and *Steinernema*, which are found in soils throughout most parts of the world, are parasitic to a broad range of insect

*Corresponding author: E-mail address: sjohnson@sun.ac.za
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pests (Malan & Hatting, 2015). Each genus is associated with a unique symbiotic bacterium – *Photorhabdus* in the case of heterorhabditids, and *Xenorhabdus* in the case of steinernematids (Ehlers, 2001), and together the EPNs and their associated bacteria are highly successful in parasitizing and killing their insect hosts. Upon encountering a suitable host, the free-living and non-feeding infective juveniles (IJ) enter the host insect through its natural openings, like the mouth, spiracles or anus (Griffin *et al*., 2005). The bacteria rapidly replicate within the nutrient-rich haemolymph of the host, generating various toxins, as well as a variety of primary and secondary metabolites that kill the host by means of inducing lethal septicaemia within 48 h of infection (Griffin *et al*., 2005).

The advantageous attributes of *Heterorhabditis* and *Steinernema* species for effective biological control include high virulence and the ability to actively seek out well-hidden life stages of insects (Lacey & Georgis, 2012). Additionally, they are compatible with commercial rearing and application techniques (Shapiro-Ilan *et al*., 2012). The entomopathogenic activity of both locally isolated steinernematids and heterorhabditids has been documented against a broad spectrum of insect pests in diverse habitats in South Africa (Hatting *et al*., 2009; Malan *et al*., 2011; Hatting & Malan 2017; Malan & Ferreira, 2017). However, their effectivity against *A. trimenii* is yet to be tested.

Entomopathogenic fungi (EPF), including *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) and the *Metarhizium anisopliae* (Metschnikoff) (Hypocreales: Clavicipitaceae) complex, are ubiquitous microorganisms that attack a variety of arthropods by means of inducing acute mycosis (Barta, 2010). The EPF can rapidly disperse horizontally among host populations by means of aerially produced conidia, and by infecting their host through penetration of the cuticle with germ hyphae (Bidochka & Small, 2005; Barta, 2010). Identification of the EPF generally relies on their physical appearance on culture media, combined with molecular identification. Morphologically, *B. bassiana* cultures are a powdery white-cream colour, turning yellow with age, whereas *M. anisopliae* cultures are varying shades of green (Coombes, 2012). The virulence of both *B. bassiana* and *M. anisopliae* is mainly a factor of the ability of the conidia to penetrate the insect's cuticle. Death of the insect host is then brought on as a result of a combination of such effects as toxinosis, general obstruction due to hyphal growth, and nutrient depletion (Wraight *et al*., 2007). Both fungal species are considered safe to vertebrates and, although they are known to have a wide host range, different strains tend to have restricted host ranges, making them suitable for use in biological control programmes (Zimmermann, 2007a, 2007b; Hatting *et al*., 2019). Additionally, the species concerned are easy and relatively inexpensive to cultivate on artificial media, which is an advantageous trait in respect of commercialisation (Kaya & Lacey, 2007). Both fungal species are known to target and successfully infest the larval and pupal stages of numerous lepidopteran pests (Nguyen *et al*., 2007; Coombes, 2012; Oliveira *et al*., 2012). However, their effectivity against *A. trimenii* has not been tested.

The current study investigated the potential of two local EPN species and two commercially available EPF species to infect *A. trimenii* under laboratory conditions and to determine their feasibility for use as part of an integrated pest management (IPM) programme directed at the management of the pest.

MATERIALS AND METHODS

Source of pupae and larvae

Both the pupae and the larvae of *A. trimenii* were collected from demarcated field sites on two table grape farms in the Northern Cape province, South Africa. Both sites consisted of netted blocks of table grapes. A block with table grape variety Thompson Seedless was used on Farm A, and a block with table grape variety Sugraone was used on Farm B. Pupae were collected during the winter months of May 2017 and June 2018 from soil mounds surrounding the vines, which were excavated to a depth of 5 cm and a radius of 60 cm. The pupae were stored in a thin layer of moistened soil in 2 L plastic containers and kept in a growth chamber at 25°C. Larval instars at various stages of development were collected in January 2018 from the top and bottom leaves of the vines. The larvae, which ranged from 1.5 cm to 4.5 cm in length, were stored in 2 L plastic containers, provided with a mesh-covered hole in the lid so as to ensure adequate ventilation, and they were given vine leaves on which to feed. The containers were stored at room temperature.

Source of nematodes and fungi

The local EPN species used in the study, *Heterorhabditis noenieputensis* Malan, Knoetze & Tiedt and *Steinernema yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, were obtained from previous surveys and stored in Stellenbosch University's nematode collection (Malan *et al*., 2006, 2011, 2014). Infective juveniles (IJs) of the two species were cultured *in vivo* at room temperature, making use of the last instar of the greater wax moth larvae, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) (Griffin *et al*., 2005). The rearing, harvesting and quantification of the IJs of both nematode species were conducted according to the methods described by Kaya and Stock (1997). Within the first week of emergence, the IJs were harvested and stored in horizontally positioned, vented 500 ml culture flasks containing approximately 100 ml of distilled water. The nematodes were used within one week after harvesting. The nematode concentrations used against the pupae and larvae were calculated according to the method of Navon and Ascher (2000).

Two commercial fungal isolates, *Beauveria bassiana* (EcoBb strain R444) and *Metarhizium anisopliae* (ICIPE 69), were received from two South African manufacturers, Plant Health Products and Real IPM, respectively. Both products were used at the concentrations stipulated on their respective labels.

Bioassay protocol for nematodes

Pathogenicity screening was conducted using six-well bioassay plates (flat-bottom, Nunce, Cat. No. 144530, Thermo Fisher Scientific [Pty] Ltd, Johannesburg, Gauteng, South Africa), with each well lined with filter paper (25 mm diameter). Each well of the bioassay plates was inoculated

with 50 µl of an adjusted IJ suspension, using a Pipetman® micropipette. A single insect was added to each inoculated well, which was then covered with a lid. An identical replicate control for each treatment was prepared on the day of screening, with 50 µl of distilled water only added to each well. The bioassay plates were then placed in 2 L plastic containers, lined with moistened paper towels and closed with a lid, to ensure the maintenance of high levels of humidity (RH ± 95%) using iButtons (iButton Link LCC – temperature and humidity data loggers). The containers were kept in the dark at 25 ± 2°C for 48 h. After two days, the insects were removed from the inoculated well plates and examined.

Pathogenicity of entomopathogenic nematodes

Pupae

The pathogenicity of *S. yirgalemense* and *H. noenieputensis* to the pupal stage of *A. trimenii* was tested at a concentration of 200 IJs/50 µl per pupa, using the bioassay protocol described above. For each EPN isolate, five six-well bioassay plates were used ($n = 30$), together with replicate controls for each treatment ($n = 30$). After 48 h, mortality was assessed by holding each pupa against a heated hotplate for 15 sec. The pupae that showed movement in response to the heat were considered alive and uninfected, while those that showed no movement were dissected and examined under a microscope for nematode infection. The experiment was repeated on a different test date with a fresh batch of nematodes, resulting in two replicate tests for each EPN tested.

Larvae

Larvae of *A. trimenii*, varying in length from 0.5 mm to 3.0 mm, were collected from vineyards (Morris, 2019). The pathogenicity of *S. yirgalemense* and *H. noenieputensis* to these larvae was tested at a concentration of 100 IJs/50 µl per larva, according to the described bioassay protocol. For each EPN isolate, four six-well bioassay plates were used ($n = 24$), with a control per EPN isolate being prepared ($n = 24$) on the day of screening. The pathogenicity was recorded by means of evaluating the mortality caused by nematode infection. After the 48 h exposure period, the larvae were rinsed with distilled water and the dead specimens were transferred to clean Petri dishes (90 mm diameter) lined with filter paper, and moistened with 800 µl of distilled water. The Petri dishes were sealed with Parafilm® and placed in a dark growth chamber for a further 48 h to encourage nematode development. All dead larvae were dissected and the presence of developing nematodes confirmed visually. The experiment was repeated with a fresh batch of nematodes on a different test date.

Bioassay protocol for fungi

The virulence of *B. bassiana* and *M. anisopliae* to *A. trimenii* pupae and larvae was assessed by means of a dipping bioassay. A conidial suspension of EcoBb (*B. bassiana* formulation) and Met69 (*M. anisopliae* formulation) was prepared by adding 0.5 g EcoBb and 0.2 ml Met69 to 500 ml distilled water, respectively. The insects were dipped in the 500 ml conidial suspensions for 30 sec. Excess suspension was removed by waiting for any surplus droplets to fall.

Six-well bioassay plates lined with filter paper were used to conduct the bioassays. The dipped insects were placed onto the filter paper of each well, and water was added to the filter paper (with no free water being allowed to remain), depending on the level of moisture obtained from each dipped insect. As controls, the insects were dipped in distilled water before being placed in their respective wells. The bioassay plates were placed in 2 L plastic containers lined with moistened paper towels, and closed with a lid to ensure the maintenance of high levels of humidity. The containers were kept in the dark at 25 ± 2°C for five days. After the set period of time had elapsed, the insects were removed from the bioassay plates and cleaned of external fungi by being dipping separately into a series of six Petri dishes containing 5% sodium hypochlorite (NaOCl), distilled water, and a 70% ethanol solution, followed by an additional three Petri dishes containing distilled water. Each dip lasted for 30 s.

Pathogenicity of entomopathogenic fungi

Pupae

For the virulence screening of each EPF isolate, five six-well bioassay plates were used (n = 30), with the control pupae (n = 30) following the bioassay protocol for the fungi described above. After five days of exposure, and subsequent washing, the pupae were transferred to 90 mm diameter Petri dishes containing a selective medium of Sabouraud Dextrose Agar (SDA) to encourage mycosis. After 10 days of possible infection, the pupae were examined visually for fungal growth. The mycosis of both isolates was recorded by observing the characteristic coloration of the conidia on the agar plates (with *B. bassiana* displaying a white mass of conidiophores and *M. anisopliae* displaying a green mass of conidiophores). Mortality was assessed by holding each individual pupa against a heated hotplate and observing any signs of movement. The pupae responding to the emitted heat were considered to be alive and uninfected.

Larvae

The virulence of both EPF isolates to the various sizes of field-collected *A. trimenii* larvae, which varied between 0.5 mm and 3.0 mm in length (Morris, 2019), was evaluated by following the dipping bioassay protocol, as described. Four six-well bioassay plates ($n = 24$) and an identical control ($n = 24$) were used per EPF isolate. After five days of exposure to EPF, dead larvae were removed from the bioassay plates and transferred to Petri dishes containing SDA to encourage mycosis. The Petri dishes were sealed in 2 L plastic containers were lined with moistened paper towels, and then placed in a growth chamber at 25 ± 2°C for a further 10 days. The mycosis of both isolates was recorded by noting the number of dead larvae, overt mycosis and the colour of the conidia.

RESULTS

Pathogenicity of entomopathogenic nematodes

Pupae

In all the bioassays, zero mortality and infection by EPNs were obtained against the pupal stage of *A. trimenii*. The pupae treated with both EPN isolates eventually emerged as adult moths after ± six months, further proving that both of

the isolates had failed to infect the pupae. No mortality was observed in the control group.

Larvae

In all the bioassays, both S. *yirgalemense* and *H. noenieputensis* caused 100% mortality after 48 h of exposure to the different larval instars of *A. trimenii* (ranging from 1.5 cm to 4.5 cm in length), at a concentration of 100 IJs/insect. No control group replicates showed mortality.

Pathogenicity of entomopathogenic fungi

Pupae

Similarly, screening *B. bassiana* and *M. anisopliae* against *A. trimenii* pupae showed zero mortality. Two pupae treated with *M. anisopliae* and one pupa treated with *B. bassiana* were assumed to be dead, as no movement was observed when they were held against the heated hotplate. However, upon inspection under the microscope, all three pupae were found to have been damaged previously, possibly as a result of excavation in the field during sample collection. Therefore, 28 of the 30 pupae treated with *M. anisopliae* were confirmed to be alive after the incubation period, and 29 of the 30 pupae treated with *B. bassiana* were confirmed to be alive after treatment. The characteristic coloration of fungal spores for each isolate was found to be lacking when the SDA plates were examined for mycosis.

Larvae

All the larvae of *A. trimenii* treated with the commercial products of *M. anisopliae* and *B. bassiana* died within five days after inoculation. After transferring the cadavers to SDA plates, the larvae treated with *M. anisopliae* showed overt mycosis after a further five days, with green fungal spores, which are typical of *Metarhizium* infection. No mortality was observed in the control group. However, the *A. trimenii* cadavers of *B. bassiana*-treated larvae showed a red/yellow colouration, with no visible mycosis. No mortality was observed in the control group.

DISCUSSION

A biological assay is the starting point of any investigation in which virulence is of importance, as it allows for the removal of factors that could reduce virulence towards the target host. Both *S. yirgalemense* and *H. noenieputensis* were found to be highly virulent, causing 100% mortality of the larval stages of *A. trimenii*. The results also indicate that inoculation with lower nematode concentrations should be applied in future studies, as this could indicate the difference in virulence between different EPN species. Success in using EPNs to control the soil life stages of other grapevine pests, including false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae) (Malan & Moore, 2016; Steyn *et al*., 2019a, 2019b); fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (James *et al*., 2018); and weevils, *Phlyctinus callosus* (Schönherr) (Coleoptera: Curculionidae) (Ferreira & Malan, 2014; Dlamini *et al*., 2019), has resulted in research being undertaken on mass culturing and the formulation of local entomopathogenic nematodes (Ferreira *et al*., 2016; Dunn *et al*., 2020). The pathogenicity of *S. yirgalemense* to *A. trimenii* larvae further emphasises its potential to control more than one target pest, which should enhance its acceptability by, and usefulness for, farmers.

The screening of both EPF isolates against the larvae of *A. trimenii* resulted in a mortality of 100%. At 10 days post-treatment, the SDA plates containing individuals treated with *M. anisopliae* showed the typical characteristic of overt mycosis with the development of green spores. However, in the case of *B. bassiana*, incubation of insect cadavers on SDA plates did not result in overt mycosis, even though no mortality occurred in the control larvae treated with water only. Further investigation to confirm toxic mycosis due to infection by *B. bassiana* is needed. These results also indicate that the application of these two biologicals in combination, to obtain a possible additive or synergistic effect, is worth investigating (Anbesse *et al*., 2008).-

Although both EPF isolates showed 100% mortality against the larval stages of *A. trimenii*, the pupal stage showed no susceptibility. The initial observations – of external sporulating fungal growth at the location of the conidial applications on the pupae, but no signs of subsequent infection/death – resemble the results obtained by Boucias and Latgé (1988) and Sitch and Jackson (1997) in the case of resistant and susceptible aphids. The pupal cuticle of *A. trimenii* provides a higher level of protection from soil-dwelling fungi than the cuticle of the arboreal larval stage.

Anand *et al*. (2009) established that the pupae of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) that resisted infection took two to five days longer to emerge than the pupae not exposed to the fungus. Additionally, Hafez *et al*. (1997) found that the pupae of the potato tuber moth, *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae), which emerged successfully despite exposure to *B. bassiana*, showed reduced fecundity. However, the indirect effects of EPF isolates on *A. trimenii* were not assessed in the present study. Despite failure to kill the pupae, the findings of this study provide future scope for testing the indirect effects of both *M. anisopliae* and *B. bassiana* on *A. trimenii* pupae. Control at the pupal stage is desired, because it would potentially significantly reduce the number of egg-laying adults and the size of subsequent *A. trimenii* populations. Testing all the EPN and EPF isolates against the prepupal stage of *A. trimenii* should be the next step in screening. Infection can possibly occur during the short window period that the last larval instar spends in the soil prior to pupation, and during the time taken by the newly eclosed moths to emerge from the soil. During the prepupal period, the cuticle tends to be softer and more malleable than the heavily sclerotized cuticle of the fully formed pupa, which makes it more challenging for the IJs to penetrate and infect the host. Kaya and Hara (1980) showed that the prepupal stage of *G. mellonella*, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) and *Mythimna unipuncta* Haworth (Lepidoptera: Noctuidae) was the most susceptible stage, exhibiting the highest mortality across all tested EPNs.

The results obtained in the present study provide useful information on the potential of EPNs and EPF to control *A. trimenii*. Limitations of the study were that a laboratory culture was not available and specimens could only be hand collected in untreated vineyards during the growing season.

All research was done on site or under quarantine conditions at the Plant Quarantine Station, Stellenbosch, South Africa. However, from the findings it can be concluded that the two local EPN species are good potential biological control agents against the larval stages of *A. trimenii*, but cannot be used to control the pupal stage, which showed zero mortality.

Applying EPNs and EPF to target the prepupal stage in the open soil cocoon (Morris *et al*., 2020), as well as targeting the newly eclosed adults moths, such as found in the case of false codling moth (Malan *et al*., 2011), holds potential for controlling the population at a time when there is no threat of damage to grapes. The ability of the EPN and EPF isolates to perform effectively under field conditions remains to be tested. Future research should be directed at investigating the effect of other EPF isolates, which are available as commercial products, either individually or in combination with EPNs as potential biological control agents against *A. trimenii*. The application of biologicals to hot spots of *A. trimenii* infestations will prevent the negative effect of chemical applications in grapevines and the disruption of a successful IPM programme.

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